Results: We have monitored the efficiency of chondrocyte depletion and effects on ECM recovery by quantification of dsDNA, glycosaminoglycans (GAGs), and protein within the hypotonic solution. While the majority of proteins were found in the guanidine-HCl extract as expected, using the discovery approach and sequential extraction, we identified several unique proteins from the extraction residue, such as fibrillin-1 and collagen IX. The extractability of proteins using guanidine-HCl was calculated for approximately 40 proteins using quantitative proteomics and MRM.
Conclusions: We have optimized the protocol to minimize confounding by intracellular proteins and managed to perform mass spectrometry analysis targeting both extractable cartilage proteins and the cartilage residue. Our results suggest that although conventional guanidine-HCl extraction is capable of extracting proteins from cartilage, the adaptations of this method provide a means of analyzing the cartilage ECM that is not confounded by intra-cellular protein and that can account for the wealth of collagen and collagen associated proteins in the cartilage residue after guanidine extraction. Such a method is essential for conducting comprehensive proteomic analysis of the cartilage in health and disease.

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AUTOPHAGY ACTIVATION PROTECTS FROM MITOCHONDRIAL DYSFUNCTION IN HUMAN CHONDROCYTES

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Purpose: A common feature of osteoarthritis (OA) and aging-related diseases, is the progressive accumulation of damaged macromolecules leading to cell dysfunction and death. Autophagy, a key pathway of cellular homeostasis by removing such damaged molecules and organelles, including mitochondria, has a protective and survival-promoting function. Recent studies indicate that autophagy activity in the articular cartilage is defective in aging and OA, contributing to the accumulation of damaged macromolecules. In addition, there is increasing evidence that mitochondrial dysfunction plays a critical role in accelerating the aging process, and several lines of evidence associate mitochondrial dysfunction to OA cartilage. The objective of this study is to determine whether activation of autophagy protects from mitochondrial dysfunction in human chondrocytes.
Methods: Human chondrocytes were treated with Rotenone (10 μg/ml), Antimycin (40 μg/ml) and Oligomycin (10 μg/ml), a mitochondrial respiratory chain (MRC) inhibitors of complex I, III and IV, respectively. Mitochondrial function and cell death were evaluated by Flow Cytometry (FC), Fluorescence Microscopy (FM). Autophagy activation was analyzed by determination of LC3-II, a main marker of autophagy activation by Immunofluorescence (IF) and Western Blot (WB). To investigate whether autophagy protects from mitochondrial dysfunction, autophagy was induced by mTOR inhibition, using mTORC1 selective inhibitor Rapamycin (Rapa, 10 μM) and mTORC1 and mTORC2 inhibitor Torin 1 (50 nM). The effects on autophagy, mitochondrial function and chondrocyte viability were analyzed by IF, WB, FC and FM. Functional studies were performed by genetic approach using siAtg5, a essential autophagy marker for autophagosome formation, to evaluate the role of autophagy on mitochondrial dysfunction.
Results: Mitochondrial dysfunction was induced by 6 h treatment with MRC inhibitors, which significantly decreased mitochondrial membrane potential ($\Delta\psi m$) (ROT: 26.17 ± 5.9; AA: 18.21 ± 3.28; Oligo: 41.74 ± 7.59, expressed as % vs control; $^*p < 0.01$). These results are consistent with increased ROS production (26.8%, 44.6% and 25.7% for ROT, AA and Oligo, respectively; $^*p < 0.001$) and cell death by apoptosis at 12 h (Control: 13.56 ± 1.83; ROT: 33.66 ± 5.55; AA: 29.05 ± 4.262; $^*p < 0.05$). Autophagy activity determined by LC3-II was increased at short incubation times, perhaps acting as an early response to stress and then decrease in a time dependent manner. To evaluate whether autophagy regulates mitochondrial dysfunction, chondrocytes were pretreated with Rapa and Torin 1 for 4 h and then treated with the MRC inhibitors for 12 h. The results show an increase in LC3 expression compared to MRC inhibitors alone. Furthermore, autophagy inducers Rapa and Torin1 increased $\Delta\psi m$ (Rapa: 125.8 ± 20.74; Rapa+ROT: 44.67 ± 10.37; Rapa+AA: 30.71 ± 5.949; Rapa ± Oligo: 108.5 ± 55.03 and Torin 1: 90.34 ± 9.17; Torin 1+ROT: 35.24 ± 2.7; Torin 1+AA: 41.98 ± 6.49; Torin 1+Oligo: 98.19 ± 16.81; $^*p < 0.05$), decreased ROS production ($^*p < 0.05$) and reduced cell death, suggesting a protective effect of autophagy activation on pharmacologically induced mitochondrial dysfunction. Importanly, blocking autophagy by siAtg5 showed a significant dysfunctional changes in the $\Delta\psi m$ and ROS production ($^*p < 0.05$), indicating the essential role of autophagy in mitocondrial function.
Conclusions: These data identify autophagy activation as a protective mechanism from mitochondrial dysfunction in human chondrocytes. Pharmacological interventions that enhance autophagy may have chondroprotective activity in pathological articular cartilage with defects on mitochondrial function.

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SMOC2 MODULATES CHONDROGENESIS BY INTERFERING WITH WNT AND BMP SIGNALING PATHWAYS

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Purpose: Osteoarthritis (OA) is a highly disabling pathology of the bone-cartilage unit, to which both patient-dependent (weight excess, trauma···) and genetic factors contribute. A proteomic analysis of OA cartilage revealed an increase in SPARC-related modular calcium binding protein-2 (SMOC2). SMOC2 was originally identified by isolation from a chondrogenic extract of articular cartilage together with GDF5 and FRZB, other proteins associated with the pathogenesis of OA. As aspects of endochondral bone formation are seen in OA (loss of stable phenotype, hypertrophy), we investigated the role of SMOC2 in chondrogenesis.
Methods: Three independent stable clonal colonies of the ATDC5 chondrogenic cell line, control (empty vector/GIPZ) and SMOC2 overexpressing (SMOC2+) or knocked-down cells (SMOC2-), were used. Clones were cultured as high density micromasses (2 x 105 cells/10 μl). Chondrogenesis was induced by culturing cells for 14 days (D) in DMEM/F12 + 5% FBS + ITS [insulin (10 μg/ml), transferrin (10 μg/ml) and sodium selenite (30 nM)]. On D14, cells were switched to mineralization medium (αMEM + 5% FBS + ITS) to investigate the mineralization phase of chondrogenesis. mRNA expression of typical chondrogenesis markers (Aggrecan (Agg), type II (Col2a1) and X (Col10a1)) collagens were assessed by quantitative RT-PCR. Likely, we assessed mRNA expression of Wnts (-3a, -4, -5a, -5b, -11) and BMPs (-2, -4, -6, -7). Quantification of Alcian Blue, Safranin O, Sirius red and Alizarin red staining were used to evaluate proteoglycans, collagens and mineralized content respectively. Western blot was used to check the activation of BMP and Wnts pathways. Cell viability was checked with Live/Dead® assay.
Results: In SMOC2+ cells, during the early (D1-7) and late proliferation phase (D7-14), Col2a1 and Agg mRNA were less increased compared to controls. Safranin O and Alcian blue staining were also less upregulated in SMOC2+. This was consistent with a lower activation of Smad 1/5/8 in SMOC2+. Col10a1 mRNA increased as expected from D14 onwards, although it was significantly lower in SMOC2+ compared to control cells. SMOC2 overexpression also affected Wnt mRNA levels, notably by reducing Wnt11. During mineralization phase (D14 to D21), Col10a1 mRNA was strongly increased, but to a lesser extent in SMOC2+, and alizarin red was much stronger in controls. The SMOC2- cells exhibited opposite features as SMOC2+. In the D1-D14 phases, Collagen and Proteoglycan content was higher than in controls, alike Col2a1 mRNA level. In the D14-21 phase, these differences were maintained, and Col10a1 mRNA level was higher in SMOC2-, and a trend was found for a higher mineralization content in SMOC2-. No difference was detected in cell viability for both SMOC2+ and SMOC2- compared to their respective controls.
Conclusions: SMOC2 modulates the chondrogenesis process, by affecting the BMP-Smad and Wnt signaling pathways.

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GSK3β INHIBITION INDUCES TERMINAL DIFFERENTIATION AND EXTRACELLULAR MATRIX REMODELLING IN HUMAN OSTEOARTHRITIC ARTICULAR CHONDROCYTES

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